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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/00, 9/00, 11/10, 15/00, A61K 49/00, G01N 31/00	A1	(11) International Publication Number: WO 99/35241 (43) International Publication Date: 15 July 1999 (15.07.99)
(21) International Application Number: PCT/US99/00401 (22) International Filing Date: 8 January 1999 (08.01.99) (30) Priority Data: 60/070,727 8 January 1998 (08.01.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/070,727 (CON) Filed on 8 January 1998 (08.01.98) (71) Applicant (for all designated States except US): RHONE-POULENC RORER PHARMACEUTICALS INC. [US/US]; 500 Arcola Road, P.O. Box 1200, Collegeville, PA 19426-0107 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ROUY, Didier [FR/FR]; 7, résidence Plein Sud, F-94320 Thiais (FR). DUVERGER, Nicolas [FR/FR]; 1, rue Martel, F-75010 Paris (FR). EM-MANUEL, Florence [FR/FR]; 63 rue Heurtault, F-93300 Aubervilliers (FR). DENEFFLE, Patrice [FR/FR]; 45, avenue des Fusilles de Chateaubriand, F-94100 Saint Maur (FR). HOUEBINE, Louis-Marie [FR/FR]; 9 bis, rue du		Haras, F-78530 Buc (FR). VIGLIETTA, Celine [FR/FR]; Bâtiment B3, Résidence Gauthier de Clagny, 59, avenue des Etats-Unis, F-78000 Versailles (FR). RUBIN, Edward [US/US]; 1436 Josephine Street, Berkeley, CA 94703 (US). HUGHES, Steven, D. [US/US]; 389 Somerset Road #3, Oakland, CA 94611 (US). (74) Agents: BARRON, Alexis et al.; Synnestvedt & Lechner LLP, 2600 Aramark Tower, 1101 Market Street, Philadelphia, PA 19107-2950 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: A TRANSGENIC RABBIT THAT EXPRESSES A FUNCTIONAL HUMAN LIPOPROTEIN (A) (57) Abstract A transgenic rabbit which has in its genomic DNA sequences that encode apolipoprotein (a) and apolipoprotein B polypeptides which are capable of combining to produce lipoprotein (a), a process for creating such a rabbit, and the use of the rabbit to identify compounds which are effective in the treatment of human diseases which are associated with, induced and/or exacerbated by Lp(a) expression.		

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**TRANSGENIC RABBIT THAT EXPRESSES
A FUNCTIONAL HUMAN LIPOPROTEIN(A)**

This invention was made with government support under grant PPG HL18574, awarded by the National Institutes of Health, and Contract No. DE-AC03-76SF00098 between the U.S. Department of Energy and the University of California. The U.S. government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is based on and claims priority to U.S. provisional Application No. 60/070,727, filed January 8, 1998.

FIELD OF THE INVENTION

The present invention relates to models of human diseases and to methods of using these models for identifying compounds effective for the treatment of these diseases. In particular, the present invention represents a new experimental model for the *in vivo* analysis of human lipoprotein (a) [Lp(a)] and the development of therapeutic strategies to reduce the health risks associated with high levels of this lipoprotein.

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Dyslipoproteinaemias are disorders in the metabolism of the lipoproteins which are responsible for transporting lipids such as cholesterol and triglycerides in the blood and the peripheral fluids. Dyslipoproteinaemias consequently are associated with life-threatening diseases linked to hypercholesterolaemia, hypocholesterolaemia or hypertriglyceridaemia, such as atherosclerosis.

Atherosclerosis is a complex, polygenic disease which is defined, on the histological plane, by deposits (lipid or fibro-lipid plaques) of lipids and other blood derivatives in the wall of the large arteries (aorta, coronary arteries and carotid). The plaques, which are more or less calcified according to the progress of the disease, can be associated with lesions and are linked to the accumulation, in the arteries, of fatty deposits which essentially consist of cholesterol esters. The plaques are accompanied by a thickening of the arterial wall together with hypertrophy of the smooth muscle, the appearance of foam cells and the accumulation of fibrous tissue. The plaques are raised on the arterial wall resulting in a stenosis, that is, a narrowing or stricture of the artery. In the worst-affected patients, this stenosis is responsible for the vascular occlusions, such as atheroma, thrombosis and embolisms. Accordingly, excess accumulation of cholesterol (hypercholesterolaemias) can lead to very serious cardiovascular diseases such as infarction, sudden death, cardiac decompensation, cerebrovascular diseases, and the like.

It is particularly important, therefore, to have immediately available treatments which diminish, in some disease situations, the levels of plasma cholesterol or stimulate the efflux of cholesterol (reverse transport of cholesterol) from the peripheral tissues in order to unload the cells which have accumulated the cholesterol in the context of forming an atheroma plaque. Cholesterol is transported in the blood by a variety of lipoproteins including the low density lipoproteins (LDL) and the high

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density lipoproteins (HDL). The LDLs are synthesized in the liver and are responsible for supplying the peripheral tissues with cholesterol. By contrast, the HDLs pick up cholesterol in the peripheral tissues and transport it to the
5 liver where it is stored and/or broken down.

Numerous studies have correlated elevated plasma levels of lipoprotein (a) [Lp(a)] with increased incidence of cardiovascular disease and stroke (reviewed in Utermann, G., *Science* (1989) 246, 904-910; Maher, V. M. G., & Brown, B. G., *Curr. Opin. Lipidol.* (1995) 6, 229-235). Lipoprotein(a)
10 is a complex particle composed of a lipid moiety and two disulfide-linked subunits: apolipoprotein B-100 (apoB-100) and apolipoprotein(a) [apo(a)]. The presence of apo(a), a hydrophilic glycoprotein structurally related to plasminogen,
15 distinguishes Lp(a) from low density lipoprotein (LDL) and confers its characteristic biological and physical properties.

Apolipoprotein AI [apo(a)] is a protein which consists of 243 amino acids and which is synthesized in the form of a
20 preproprotein of 267 residues, having a molecular mass of 28,000 daltons. In humans, it is synthesized specifically in the liver and the intestine and it constitutes the essential protein of the HDL particles (70% of their weight in proteins). It is abundant in the plasma (1.0-1.2 g/l). While
25 its activity which is best characterized from the biochemical point of view as activation of lecithin-cholesterol acyltransferase (LCAT), a large number of other activities are attributed to it, such as, in particular, stimulation of the efflux of cellular cholesterol. The physiological role of
30 apolipoprotein AI appears to be counterbalanced by apolipoprotein AII since, in humans, the ratio of the two plasma concentrations (AII/AI) is very closely correlated with coronary risk. Apolipoprotein AI plays a major role in resistance to atherosclerosis, which role is probably linked
35 to the reverse transport of cholesterol, since expression of this apolipoprotein alone in transgenic mice results in the surface of the lipid deposits in the aorta being reduced

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40-fold as compared with control mice (Rubin et al. 1993 Science, vol 365 p 762). Its gene, which is 1863 bp in length, has been cloned and sequenced (Sharpe et al., Nucleic Acids Res. (1984) 12(9):3917). A variety of proteinaceous products having apolipoprotein AI type activity have been identified. These products include a variety of natural variants which have been described in the prior art, as shown in Table 1.

Table 1: Naturally-Occurring Variants of Apolipoprotein AI

Variant:	Mutation	Variant	Mutation
Milano	Arg173Cys	Norway	Glu136Lys
Marburg	Lys107 ⁻		Pro165Arg
Munster2B	Ala158Glu		Pro3His
Giessen	Pro143Arg		Arg10Leu
Munster3A	Asp103Asn		Gly26Arg
Munster3B	Pro4Arg		Asp89Glu
Munster3C	Pro3Arg		Lys107Met
Munster3D	Asp213Gly		Glu139Gly
Munster4	Glu198Lys		Glu147Val
Yame	Asp13Tyr		Ala158Glu
	Asp213Gly		Glu169Gln
			Arg177His

Apolipoprotein B-100 (apoB) is the major protein constituent of very low density lipoproteins (VLDL), low density lipoproteins (LDL) and lipoprotein Lp(a). This protein is the physiological ligand of the LDL receptor, and its plasma concentration is positively correlated with the development of atherosclerosis (Brunzell et al. 1984 Arteriosclerosis 4, 79-93). ApoB-100 is one of the largest known proteins, with a mass of 550 kDa and containing 4536 amino acids (Chen et al. 1986 J. Biol. Chem, 261, 12919-21). This apolipoprotein is only synthesized in the liver. Its plasma concentration is 1.0-1.2 g/l. ApoB-100 plays the major role in transporting cholesterol which is synthesized in the liver through the plasma to the other cells of the organism. Another version of apoB, i.e. apoB-48, is present in the

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chylomicrons. In humans, apoB-48 is synthesized in the intestine. ApoB-48 has a mass of 260 kDa and contains 2152 amino acids which linearly correspond to 48% of the N-terminal end of apoB-100 (Powell et al., 1987, Cell 50, 831-40). Since the C-terminal moiety of apoB-100 contains the zone for binding the apoB-100 to the LDL receptor, apoB-48 does not attach to this latter receptor and behaves in a different manner metabolically.

To study the disease states relating to dyslipoproteinaemias, it is advantageous to have available an animal model which expresses a protein or a protein complex which is associated with a risk for a disease which is linked to dyslipoproteinaemias. Such an animal model would be particularly advantageous for understanding these diseases and, more specifically, the regulatory mechanisms which these proteins or protein complexes initiate. This would make it possible to test, rapidly and *in vivo*, a considerable number of therapeutic agents or compounds for the purpose of detecting a potential activity associated with the expression of the proteins. Furthermore, such a model would be of interest for developing novel therapeutic methods for treating these types of diseases, such as methods which are based on gene therapy. The *in vivo* analysis of Lp(a), an independent atherosclerosis risk factor in humans, has been limited in part by its restricted distribution among mammals. Apo(a) is naturally present exclusively in old world monkeys, humans, and one non-primate species, the European hedgehog. Such limited distribution of apo(a) among mammals has limited studies of its *in vivo* properties.

Accordingly, the present invention relates to animal models of disease states involving Lp(a), including atherosclerosis, to enable screening and identification of compounds for the treatment of these diseases, in particular, atherosclerosis.

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Reported Developments

Generally speaking, the murines, namely mice, rats and guinea pigs, are the most widely used animal models. They are easy to manipulate and inexpensive. Unfortunately, these small mammals are not always compatible with the intended application because they are not always representative of humans and their metabolism. Chimpanzees are used for testing therapeutic agents and vaccines directed against various diseases, including AIDS and cancer. However, the very substantial cost incurred in using chimpanzees as a model system constitutes a major and compelling handicap with regard to its use.

Despite the drawbacks of a system using mice, the development of transgenic mice expressing human apo(a) cDNA provided a means to test hypotheses accounting for the effect of Lp(a) on the vasculature. Specifically, these mice have been used to examine the ability of apo(a) to promote atherogenesis by inhibition of plasmin formation and associated consequences (Lawn, et al., *Nature* (1992) **360**, 670-672; Grainger, et al., *Nature* (1994) **370**, 460-462). Studies of apo(a) transgenic mice have also led to several important insights into Lp(a) assembly, including the observation that apo(a) was unable to form a covalent association with LDL containing murine apoB (Chiesa, et al., *J Biol Chem* (1992) **267**, 24369-24374). This result, coupled with evidence for Lp(a) formation when the mice were infused with human LDL or expressed a human apoB transgene (Linton, et al., *J Clin Invest* (1993) **92**, 3029-3037; Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) **91**, 2130-2136) suggested that murine apoB lacked structural requirements necessary for Lp(a) assembly. This was not a completely unexpected finding in light of the absence of the apo(a) gene in mice and the sequence specific interactions between apo(a) and apoB believed to mediate Lp(a) assembly in humans. Two studies using site specific mutagenesis of human apoB transgenes in mice have reported localization of a single cysteine in human apoB (Cys 4326) which provides the site of attachment for

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apo(a) (Callow, M. J., & Rubin, E. M., *J Biol Chem* (1995) 270, 23914-23917; McCormick, et al., (1995) 92, 10147-10151).

Most recently, the regulation of apo(a) gene expression has been studied in transgenic mice containing a human apo(a) genomic clone (Frazer, et al., *Nat Gen* (1995) 9, 424-431). This transgene comprised the apo(a) gene along with its native promoter and cis acting elements present within the approximately 60 kb of 5' and 80 kb of 3' flanking DNA. The transgene was more efficiently expressed (i.e. all the apo[a] transgenic founder lines created containing an intact transgene expressed apo[a]) and resulted in significantly higher plasma apo(a) levels than observed in mice containing an apo(a) cDNA construct. A surprising finding in mice expressing the human apo(a) genomic transgene was the profound sex hormone-induced changes in apo(a) expression, far surpassing the magnitude of androgen and estrogen related changes observed in humans. These changes were, however, qualitatively similar in humans and transgenic mice. In both cases, these hormones lower apo(a) plasma levels.

The use of genomic clones in creating transgenic animals generally has several advantages over the use of cDNA constructs. Most importantly, transgene expression is regulated in an appropriate manner and is independent of its site of integration. This approach has been limited to the production of transgenic mice for large genes such as apo(a), however, due to the technical difficulty of manipulating extremely large genomic clones and the lower efficiency of transgenesis in other animals.

Although transgenic mice have been created containing Lp(a), the small size of the mouse and the differences in lipoprotein profiles between mice and humans has precluded various studies. Accordingly, it is desirable to identify an animal model system which is closer to man but avoids the expense associated with primate model systems.

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Thus, there is a need in the art for an appropriate animal model of Lp(a)-mediated diseases and disorders. There is a further need in the art for a transgenic animal, that expresses human Lp(a). Moreover, to be truly useful, a
5 transgenic animal must express the constituent Lp(a) proteins at a high enough level, and must permit covalent association of the constituents so as to produce physiologically relevant levels of Lp(a). To date, the technical difficulties in generating such an animal have not been overcome, until the
10 present invention.

The present invention overcomes these deficiencies of the prior art, by providing a useful transgenic animal that produces physiological levels of human Lp(a), despite the difficulties in achieving such an invention.

15 In particular, the invention relates to transgenic animals expressing both the human apolipoprotein(a) [apo(a)] and apolipoprotein B (apoB) genes. These animals express human lipoprotein(a) [Lp(a)], the complex particle composed of a lipid moiety and two disulfide-linked subunits of apo(a)
20 and apoB.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a transgenic rabbit which has in its genomic DNA sequences that encode apolipoprotein (a) and apolipoprotein B
25 polypeptides which are capable of combining to produce lipoprotein (a).

The present invention also provides a transgenic rabbit capable of producing lipoprotein (a) wherein the rabbit develops human-like atherosclerotic lesions when fed a
30 cholesterol rich diet.

The present invention also provides for transgenic rabbits capable of producing lipoprotein (a) having exogenous human DNA sequences. The DNA sequences may be selected from genomic DNAs and cDNAs.

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As stated above, the transgenic rabbits of the present invention have in their genomic DNA sequences that encode apolipoprotein (a) and apolipoprotein B. The present invention provides transgenic rabbits capable of producing
5 lipoprotein (a) having liver cells which express the DNA sequences and transgenic rabbits capable of producing lipoprotein (a) having testes cells which express the DNA sequences.

Yet another aspect of the present invention is a
10 transgenic rabbit having a stable plasma level of apolipoprotein (a) polypeptide throughout its sexual maturity.

The present invention also provides a process for creating a transgenic rabbit which is capable of expressing
15 apolipoprotein (a) and apolipoprotein B polypeptides and which comprises combining the germ line cells of a first rabbit which is capable of expressing apolipoprotein (a) with the germ cells of a second rabbit which is capable of expressing apolipoprotein B. Germ line cells include, for
20 example, eggs and sperm.

Yet another aspect of the present invention is the provision of a process for creating a transgenic rabbit which is capable of expressing apolipoprotein (a) and apolipoprotein B polypeptides and which comprises mating a
25 first rabbit which is capable of expressing apolipoprotein (a) with another rabbit which is capable of expressing apolipoprotein B.

In a preferred method for creating a transgenic rabbit capable of expressing lipoprotein (a), the rabbit expressing apolipoprotein B protein is provided by injecting a rabbit embryo with a phagemid which contains the human apolipoprotein B gene.

In a preferred method for creating a transgenic rabbit capable of expressing lipoprotein (a), the rabbit expressing

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apolipoprotein (a) is provided by injecting a rabbit embryo with a yeast artificial chromosome which contains a human apolipoprotein (a) gene.

In yet another method for creating a transgenic rabbit capable of expressing lipoprotein (a), the rabbit expressing apolipoprotein (a) is provided by introducing into a rabbit embryonal stem cell at least one human DNA fragment encoding
5 apolipoprotein (a) protein, combining the stem cell with a rabbit blastocyst and transferring the embryo to a recipient female rabbit.

In yet another method for creating a transgenic rabbit capable of expressing lipoprotein (a), the rabbit expressing
10 apolipoprotein B is provided by introducing into a rabbit embryonal stem cell at least one human DNA fragment encoding apolipoprotein B protein, combining the stem cell with a rabbit blastocyst and transferring the embryo to a recipient female rabbit.

15 In still yet another method for creating a transgenic rabbit capable of expressing lipoprotein (a), the rabbit carrying and expressing apolipoprotein (a) is provided by infecting a rabbit blastomere with a retrovirus comprising at least one human DNA fragment encoding apolipoprotein (a)
20 protein and transferring the blastomere to a recipient female rabbit.

In yet another method for producing a transgenic rabbit capable of expressing lipoprotein (a), the rabbit carrying and expressing apolipoprotein B is provided by infecting a
25 rabbit blastomere with a retrovirus comprising at least one human DNA fragment encoding apolipoprotein B protein, and transferring the blastomere to a recipient female rabbit.

The present invention also provides a method for determining whether a compound can inhibit a disease or
30 disorder linked to lipoprotein (a) expression or metabolism comprising comparing a first transgenic rabbit which is

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capable of producing human lipoprotein (a) and manifests a disease or disorder linked to human lipoprotein (a) and which has been treated with the compound, with a second transgenic rabbit which is capable of producing human lipoprotein (a) and manifests a disease or disorder linked to lipoprotein (a) and which has not been treated with the compound. The present invention also provides for utilizing this method wherein the disease or disorder is selected from the group consisting of atherosclerosis, cardiovascular disease, ischemia, stroke, restenosis, coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, thrombosis, and an undesirable lipid profile.

The present invention also provides a method for determining whether a compound can inhibit assembly of a lipoprotein (a) particle comprising comparing the level of lipoprotein (a) production in a first transgenic rabbit which has been treated with said compound and which is capable of producing human lipoprotein (a) to the level of production of lipoprotein (a) in a second transgenic rabbit that has not been treated with said compound and which is capable of producing human lipoprotein (a). The present invention also provides for utilizing this method wherein the compound is selected from the group consisting of antisense nucleic acids, and intracellular binding proteins.

The present invention provides transgenic rabbits, an appropriate animal model of lipoprotein (a)-mediated diseases and disorders. The rabbits express the constituent lipoprotein (a) proteins at a high level so as to facilitate covalent association of the constituents to produce physiologically relevant levels of lipoprotein (a). The rabbits offer significant advantages over transgenic mice, due, in part, to the larger size of the rabbit which facilitates study of vascular injury and restenosis. Furthermore, although rabbits are similar to mice in lacking apolipoprotein (a) and lipoprotein (a), the rabbit's lipoprotein profile more closely mimics that of humans with LDL as the predominant plasma protein. In addition, rabbits

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also develop well-characterized human-like atherosclerotic lesions when fed a cholesterol-rich diet.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. PCR analysis of human apoB and apo(a) in transgenic rabbits

Genomic DNA prepared from founder animals and progeny derived from crossing apo(a) and apoB transgenics was subjected to polymerase chain reaction (PCR) analysis. The apo(a) transgenic founder (sample 2) contained the complete apo(a) genomic clone as judged by amplification with four different primer sets: plasminogen 5' region (PMG 5'), apo(a) 5', apo(a) 3', and apo(a)-like gene 3' (positions indicated below diagram of the genomic clone). ApoB transgenic founders (sample 1) were detected using a single PCR reaction specific for human sequences. Analyses of representative progeny from the apo(a)/apoB matings, shown in samples 3 and 4 (apo(a) only and combined apo(a)/apoB, respectively), confirmed transgene transmission. For each primer set, apo(a) or apoB genomic clone DNA was included as a positive control (sample 5).

Figure 2. Tissue distribution of apo(a) and apoB transgene expression.

Expression of apo(a) (panel A) and human apoB (panel B) was analyzed in the indicated tissues of control (c) and transgenic (tg) rabbits by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. As a control for human apoB specificity, as well as sample quality, rabbit apoB mRNA was also amplified using species specific primers (panel C).

Figure 3. Time course of apo(a) levels in transgenic rabbits

Plasma samples were taken from male and female apo(a) transgenic rabbits at 3 time points after birth: 3, 6, and 9 months. 1 μ L of each sample was subjected to SDS-PAGE under reducing conditions and immunoblotting using antibodies raised against apo(a).

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Figure 4. Immunoblotting of transgenic rabbit plasma

Equal volumes of plasma (1 μ l) from a human apo(a) transgenic rabbit (1), a human apoB/apo(a) transgenic rabbit (2), and a human apoB transgenic rabbit (3) were size fractionated by SDS-PAGE. Gels were run under non-reducing conditions (panel A) and under reducing conditions (panels B and C). Immunoblotting was performed using antibodies raised against apo(a) (panels A and B) and against human apoB (panel C). Human plasma was included as a control. Sizes of the observed proteins were evaluated using a curve of migrating prestained molecular weight standards vs. migration distance.

Figure 5. Immunoblotting of lipoprotein fractions from transgenic rabbits

100 μ L of plasma from human apoB/apo(a) transgenic rabbits (A) or apo(a) transgenic rabbits (B) was size fractionated by chromatography on a Superose 6 column. Even numbered fractions (6-40) were subjected to SDS-PAGE under non-reducing conditions and immunoblotting using antibodies raised against apo(a). Lipoprotein size ranges, determined by cholesterol assay of the fractions, are indicated in the bar between panels A and B.

Figure 6. Protein sequence alignment of human and rabbit apoB

Protein sequence of rabbit apoB (residues 4315-4364; SEQ ID NO:7) aligned with homologous regions of human (SEQ ID NO:8), rat (SEQ ID NO:9), pig (SEQ ID NO:10) and chicken (SEQ ID NO:11) apoB. Sequence identity is indicated by capital letters. Numbering above the sequences corresponds to the human apoB sequence. The * indicates the site of apo(a) attachment to human apoB.

30

Definitions

The following defined terms are used throughout the present specification, and should be helpful in understanding the scope and practice of the present invention.

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A "polypeptide" is a polymeric compound comprised of covalently linked amino acid residues. Amino acids have the following general structure:



Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group.

A "protein" is a polypeptide which plays a structural or functional role in a living cell. Within the meaning of the present invention, the designation "lipoprotein(a) complex" is understood to cover the apolipoproteins (a) and B as well as Lp(a) and any other proteinaceous product which has an Lp(a) activity. Preferably, the term proteinaceous product denotes any mutant, fragment or peptide which possesses at least one biological property of an apolipoprotein, as well as any natural variant of the apolipoproteins. The polypeptides and proteins of the invention may be glycosylated or unglycosylated.

A "variant" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. Variants also

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include a related protein having substantially the same biological activity, but obtained from a different species.

The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, inter alia: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide or protein, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which the polypeptide or protein is fused with another polypeptide such as serum albumin. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art.

If such allelic variations, analogues, fragments, derivatives, mutants, and modifications, including alternative mRNA splicing forms and alternative post-translational modification forms result in derivatives of the polypeptide which retain any of the biological properties of the polypeptide, they are intended to be included within the scope of this invention.

A "nucleic acid" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semi-synthetic DNA. The sequence of nucleotides that encodes a protein is called the sense sequence. An 'apo(a) gene' is any gene which encodes an apolipoprotein(a) protein. An 'apoB gene' is any gene which encodes an apolipoprotein B protein. Preferred apo(a) and apoB genes encode human apo(a) and apoB proteins.

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As used herein, the term "**homologous**" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (e.g., the immunoglobulin superfamily) and homologous proteins from different species (e.g., myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667). Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity.

Accordingly, the term "**sequence similarity**" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (see Reeck et al., *supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

In a specific embodiment, two DNA sequences are "**substantially homologous**" or "**substantially similar**" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, in a particular embodiment, two amino acid sequences are "**substantially homologous**" or "**substantially similar**" when greater than about 30% of the amino acids are identical, or greater than about 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for

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example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program.

The term "**corresponding to**" is used herein to refer
5 similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "**corresponding to**" refers to the sequence similarity, and not
10 the numbering of the amino acid residues or nucleotide bases.

The present invention relates to transgenic animals, i.e., rabbits, containing genes encoding analogs and derivatives of apolipoproteins that have the same or homologous functional activity as the apolipoproteins, and
15 homologs thereof from other species. The production and use of **derivatives and analogs** related to apolipoproteins are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities
20 associated with a full-length, wild-type apolipoprotein of the invention. In particular, an apolipoprotein derivative of the invention is capable of forming a functional human Lp(a) particle.

Apolipoprotein derivatives can be made by altering
25 encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native apolipoproteins, i.e., apo(a) and apoB. As used herein, the
30 term "**apolipoprotein**" refers to apo(a) or apoB.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an apolipoprotein gene may be used in the practice of the present invention. These include, but are
35 not limited to, allelic genes, homologous genes from other

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species, and nucleotide sequences comprising all or portions of apolipoprotein genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change.

5 Likewise, the apolipoprotein derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of an apolipoprotein including altered sequences in which functionally equivalent amino acid residues are
10 substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent
15 alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and
20 methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine,
25 lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point. Particularly preferred substitutions
30 are:

- Lys for Arg and vice versa such that a positive charge may be maintained;

- Glu for Asp and vice versa such that a negative charge may be maintained;

35 - Ser for Thr such that a free -OH can be maintained; and

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- Gln for Asn such that a free CONH₂ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential
5 site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the
10 protein's structure.

The genes encoding apolipoprotein derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For
15 example, the cloned apolipoprotein gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired,
20 isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of an apolipoprotein, care should be taken to ensure that the modified gene remains within the same translational reading frame as the native apolipoprotein gene, uninterrupted by translational stop
25 signals, in the gene region where the desired activity is encoded.

Additionally, the apolipoprotein-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination
30 sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated apolipoprotein gene
35 product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed

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mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB[®] linkers (Pharmacia),
5 etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

10 Generally, the nucleic acids of the present invention are linked to one or more regulatory regions. Selection of the appropriate regulatory region or regions is a routine matter, within the level of ordinary skill in the art. "Regulatory region" means a nucleic acid sequence which
15 regulates the expression of a second nucleic acid sequence. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may include sequences of a different origin (responsible for expressing different proteins or even
20 synthetic proteins). In particular, the sequences can be sequences of eukaryotic or viral genes or derived sequences which stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions include origins of
25 replication, RNA splice sites, enhancers, transcriptional termination sequences, signal sequences which direct the polypeptide into the secretory pathways of the target cell, and promoters.

 The regulatory regions may comprise a promoter region
30 for functional transcription in endothelial cells, liver, testis, brain or other cell types in which apo(a) and apoB expression is desired, as well as a region situated in 3' of the gene of interest, and which specifies a signal for termination of transcription and a polyadenylation site. All
35 these elements constitute an expression cassette.

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"Promoters" that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoter may be naturally responsible for the expression of the nucleic acid. It may also be from a heterologous source. In particular, it may be promoter sequences of eucaryotic or viral genes. For example, it may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, it may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, CMV and RSV genes and the like. The non-viral promoter sequences which are preferably used are the ApoAI promoters or the hepatic enhancers of ApoE or the intestinal enhancers of apoCIII. Naturally, these expression sequences can additionally be modified by adding activating sequences, regulatory sequences, etc. The genomic DNA can also be included in a large-capacity expression vector such as the P1 vector or else the YAC, cosmid or plasmid vectors.

In addition, the promoter may be modified by addition of activating or regulatory sequences or sequences allowing a tissue-specific or predominant expression (enolase and GFAP promoters and the like). Moreover, when the nucleic acid does not contain promoter sequences, it may be inserted, such as into the virus genome downstream of such a sequence.

Some promoters useful for practice of this invention are ubiquitous promoters (e.g. HPRT, vimentin, actin, tubulin), intermediate filament promoters (e.g. desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (e.g. MDR type, CFTR, factor VIII), tissue-specific promoters (e.g. actin promoter in smooth muscle cells), promoters which are preferentially activated in dividing cells, promoters which respond to a stimulus (e.g. steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modulators

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and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the contents of which are incorporated herein by reference.

A regulatory region from a "heterologous source" is a
5 regulatory region which is not naturally associated with the expressed nucleic acid. Included among the heterologous regulatory regions are regulatory regions from a different species, regulatory regions from a different gene, hybrid regulatory sequences, and regulatory sequences which do not
10 occur in nature, but which are designed by one having ordinary skill in the art.

A "vector" is any means for the transfer of a nucleic acid according to the invention into a host cell. The term "vector" includes both viral and nonviral means for
15 introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. Non-viral vectors include plasmids, phagemids, BAC, PAC, YAC, P1, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. The vectors containing a nucleic acid according to the invention
20 may include plasmid, phagemid, P1, BAC, PAC, YAC or cosmid vectors. The vectors of particular interest to the invention include those that are able to accomodate large DNA sequences, (i.e., phagemids, plasmids, P1, BAC, PAC, YAC or cosmid vectors), especially in the case of genomic DNA
25 sequences pertaining to the invention. In addition to a nucleic acid according to the invention, a vector may also contain one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues,
30 duration of expression, etc.).

"Lipid profile" means the set of concentrations of cholesterol, triglyceride, lipoprotein cholesterol and other lipids in the body of a human or other animal.

An "undesirable lipid profile" is the condition in which
35 the concentrations of cholesterol, triglyceride, or

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lipoprotein cholesterol are outside of the age- and gender-adjusted reference ranges. Generally, a concentration of total cholesterol > 200 mg/dl, of plasma triglycerides > 200 mg/dl, of LDL cholesterol > 130 mg/dl, of HDL cholesterol < 5 39 mg/dl, or a ratio of total cholesterol to HDL cholesterol > 4.0 is considered to be an undesirable lipid profile. An undesirable lipid profile is associated with a variety of pathological conditions, including hyperlipidaemias, diabetes hypercholesterolaemia, atherosclerosis, and other forms of
10 coronary artery disease.

The down regulation of gene expression using antisense nucleic acids can be achieved at the translational or transcriptional level. **Antisense nucleic acids** of the invention are preferably nucleic acid fragments capable of
15 specifically hybridizing with a nucleic acid encoding apolipoprotein (a) or B or the corresponding messenger RNAs. These antisense nucleic acids can be synthetic oligonucleotides, optionally modified to improve their stability and selectivity. They can also be DNA sequences
20 whose expression in the cell produces RNA complementary to all or part of the mRNA encoding apolipoprotein (a) or B. Antisense nucleic acids can be prepared by expression of all or part of a nucleic acid encoding apolipoprotein (a) or B, in the opposite orientation, as described in EP 140308. Any
25 length of antisense sequence is suitable for practice of the invention so long as it is capable of down-regulating or blocking expression of apolipoprotein (a) or B. Preferably, the antisense sequence is at least 20 nucleotides in length. The preparation and use of antisense nucleic acids, DNA
30 encoding antisense RNAs and the use of oligo and genetic antisense is disclosed in WO92/15680, the contents of which are incorporated herein by reference.

A **"pharmaceutically acceptable carrier"** comprises diluents and fillers which are pharmaceutically acceptable
35 for methods of administration, are sterile, and may be aqueous or oleaginous suspensions formulated using suitable dispersing or wetting agents and suspending agents. The

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particular pharmaceutically acceptable carrier and the ratio of active compound to carrier are determined by the solubility and chemical properties of the composition, the particular mode of administration, and standard
5 pharmaceutical practice.

The term "physiological level" is used herein to refer to a level of protein expression, or Lp(a) production, that results in the transgenic animal possessing a lipoprotein profile closely mimicking that of humans. In particular, the
10 transgenic animal is able to develop well characterized human-like atherosclerotic lesions when fed a cholesterol rich diet. In a specific embodiment, the level of Lp(a) in the transgenic animal is within the range found in humans.

The terms "transgenic animals" and "animal" is used
15 herein to include all vertebrate animals, except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic animal" is an animal containing one or more cells bearing genetic information received, directly or indirectly,
20 by deliberate genetic manipulation at a subcellular level, such as by microinjection or infection with recombinant virus. This introduced DNA molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. The term "germ cell-line transgenic animal" refers to a
25 transgenic animal in which the genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

30 Detailed Description of the Invention

As discussed hereinabove, there is a need in the art for an appropriate animal model of Lp(a)-mediated diseases and disorders. In particular, it is important to provide an experimental model for the *in vivo* analysis of human
35 lipoprotein (a). Applicants have obtained for the first time

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in the art transgenic rabbits capable of providing lipoprotein (a).

Transgenic rabbits represent an increasingly utilized approach to the study of apolipoproteins and their impact on lipoprotein metabolism and atherogenesis (Fan, et al., 5 *Arterioscler Thromb Vasc Biol* (1995) **15**, 1889-1899; Duverger, et al., *Arterioscler Thromb Vasc Biol* (1996) **16**, 1424-1429). The advantages of this animal, compared to the mouse, relate in part to its relatively larger size, enabling 10 facile studies of vascular injury and restenosis. In addition, while rabbits are similar to mice in lacking apo(a) and Lp(a), their lipoprotein profile more closely mimics that of humans, with LDL as the predominate plasma lipoprotein (Chapman, M. J., in *Methods in Enzymology*, vol. 128: *Plasma* 15 *Lipoproteins, Part A, Preparation, Structure, and Molecular Biology* Segrest, J. P., & Albers, J. J., Eds. (Academic Press, Inc., New York, 1986), vol. 128, pp. 70-143). Rabbits also develop well characterized human-like atherosclerotic lesions when fed a cholesterol-rich diet.

20 Within the scope of the present invention, the rabbit has proved to be the appropriate animal model. A comprehensive knowledge exists of the metabolism of the rabbit and of the diseases of this animal which are linked to lipoproteins. The rabbit is an animal which is classified as 25 "LDL mammalian", that is to say the LDLs are the major transporters of plasma cholesterol as in man, contrary to rats and mice, which are animals classified as "HDL mammalian". Furthermore, a large number of genetic variations exist in the lipoproteins within the rabbit lines, such as 30 the WHHL rabbits which are deficient in LDL receptor (Watanabe heritable hyperlipidaemic rabbit) or else the "St Thomas Hospital" rabbits, which overproduce LDLs (Rosenfeld et al., 1990, *Arteriosclerosis* **10**, 680-687; Sedon et al.; 1987, *Arteriosclerosis* **7**, 113-124).

35 The present invention provides a new, relatively large animal model for studying the biological properties of Lp(a),

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in the form of a rabbit which contains both human genomic transgenes for apo(a) or apoB. The rabbits demonstrate efficient, tissue specific expression of both human transgenes and the assembly of Lp(a) particles. In addition, apo(a) covalently interacts *in vivo* with rabbit apoB and the transgenic rabbit model represents a means to characterize this novel interaction. Applicants have prepared a transgenic rabbit into whose genome has been inserted exogenous genomic DNA sequences which encode apolipoprotein(a) and apolipoprotein B proteins which form a lipoprotein(a) complex.

In addition to providing unique insights into Lp(a) assembly and apo(a) expression, several features of the Lp(a) transgenic rabbits provided by this invention represent improvements over currently available animal models for investigating Lp(a). These animals possess a lipoprotein profile closely mimicking that of humans and develop well characterized human-like atherosclerotic lesions when fed a cholesterol-rich diet. Also, due to their relatively large size, they have been successfully used in vascular injury and restenosis studies. Thus, these transgenic rabbits will provide a valuable resource to study factors affecting Lp(a) levels as well as the effects of Lp(a) on the progression of atherosclerosis and vascular disease.

Any rabbit can be used to prepare a transgenic animal of the invention. Preferably, the rabbit is a homogeneous laboratory strain, which can be obtained from any animal source such as Charles River or Jackson Laboratories (Bar Harbor, Maine). In a specific embodiment, *infra*, the rabbit is a New Zealand White rabbit. Alternatively, a Watanabe hyperlipidemic transgenic rabbit of the invention can be generated.

In a preferred embodiment, the present invention provides a process for creating the transgenic rabbit by injecting, into a rabbit embryo, exogenous genomic DNA sequences encoding apo(a) or apoB proteins which form a lipoprotein(a) complex, transferring the embryo to a recipient rabbit and, after the

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birth, checking for the presence of the genomic DNA sequences in the genome of the neonate rabbits. Apo(a) transgenic rabbits and apoB transgenic rabbits are then mated to produce the apo(a)-apoB transgenic rabbit which expresses Lp(a).

5 The process for creating the transgenic rabbit is described in more detail in Example 2 below. The implementation of such a process does not present any difficulties to the skilled person who is familiar with the techniques of microinjection, and removal and implantation of
10 embryos, as well as animal husbandry.

 The present invention especially relates to the use of a 270 kb yeast artificial chromosome (YAC) clone which contains the human apo(a) gene and a 90 kb P1 phagemid clone which contains the human apoB gene to create a transgenic rabbit
15 which expresses both transgenes. The transgenic rabbit of the present invention exhibits tissue-specific expression of both transgenes which is localized predominantly in the liver. In contrast to observations in apo(a) transgenic mice, the current invention results in apo(a) plasma levels that are
20 stable throughout sexual maturity in the rabbits. In addition, apo(a) is able to form a covalent association with the endogenous rabbit apoB, albeit with a lower efficiency than its association with human apoB. The invention of this Lp(a) transgenic rabbit model provides for new insights into
25 the structural requirements for Lp(a) assembly and regulation of apo(a) expression. In addition, these transgenic rabbits represent a new experimental model for the *in vivo* analysis of Lp(a) and the development of therapeutic strategies to reduce atherosclerosis risk associated with high levels of this
30 lipoprotein.

 Accordingly, the present invention provides a transgenic rabbit comprising both apo(a) and apoB genes or variants thereof, and the rabbit is capable of expressing these genes such that a lipoprotein(a) complex is formed.

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The genetic information present in the transgenic rabbits may be foreign to the species of animal to which the recipient belongs, (i.e. exogenous) foreign only to the particular individual recipient (i.e. exogenous), or genetic
5 information already possessed by the recipient. In the last case, the introduced gene may be differently expressed compared to the native endogenous gene.

The genes may be obtained by isolating them from genomic sources, by preparation of cDNAs from isolated RNA templates,
10 by directed synthesis, or by some combination thereof. In a preferred embodiment of the invention, the human apo(a) and apoB genes are cloned from genomic DNA, such that the transgenes' expression is regulated in an appropriate manner and is independent of its site of integration into the
15 transgenic animal's chromosomal DNA.

A transgenic rabbit according to the invention can integrate the genomic DNA sequences into all its cells or only into a certain percentage of cells; in the latter case it would be termed mosaic. In general, the genomic DNA
20 sequences are integrated into all the cells. The inserted genomic DNA sequences according to the invention encode all, or an active part, of the proteins which are involved in lipoprotein(a) complex formation and/or metabolism. These genomic DNA sequences can also contain several genes which
25 are organized, if need be, in cluster form.

Those inserted DNA sequences within the meaning of the present invention which may more specifically be cited are the genes which encode all or an active part of apolipoproteins (a), B, or a variant or a derivative of these
30 apolipoproteins, and form an Lp(a) complex.

To be expressed, a gene should be operably linked to a regulatory region. Regulatory regions, such as promoters, may be used to increase, decrease, regulate or designate to certain tissues or to certain stages of development the
35 expression of a gene. The promoter need not be a naturally

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occurring promoter. The "transgenic non-human animals" of the invention are produced by introducing "transgenes" into the germline of the non-human animal. The methods enabling the introduction of DNA into cells are generally available and well-known in the art. Different methods of introducing transgenes could be used. Generally, the zygote is the preferred target for microinjection. The use of zygotes as a target for gene transfer has a major advantage. In most cases, the injected DNA will be incorporated into the host gene before the first cleavage (Brinster, et al., (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4438-4442). Consequently, nearly all cells of the transgenic non-human animal will carry the incorporated transgene. Generally, this will also result in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is a preferred method for incorporating transgenes in practicing the invention.

Retroviral infection can also be used to introduce a transgene into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, blastomeres may be targets for retroviral infection (Jaenich, R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan et al., (1986) in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al., (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6927-6931; Van der Putten et al., (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten et al., (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6148-6152; Stewart et al., (1987) *EMBO J.* **6**:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al.,

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(1982) *Nature* 298:623-628.). Most of the founder animals will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Furthermore, the founder animal may
5 contain retroviral insertions of the transgene at a variety of positions in the genome; these generally segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo
10 (Jahner et al., (1982) *Nature* 298:623-628.).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro (Evans, M.J., et al., (1981) *Nature* 292, 154-156; Bradley, A., et al. (1984)
15 *Nature* 309, 255-258; Gossler, et al., (1986) *Proc. Natl. Acad. Sci. USA* 83, 9065-9060; and Robertson, et al., (1986) *Nature* 322, 445-448). Transgenes can be efficiently introduced into ES cells by DNA transfection or by retrovirus-mediated transduction. The resulting transformed
20 ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells colonize the embryo and contribute to the germ line of the resulting chimeric animal (For review see Jaenisch, R. (1988) *Science* 240, 1468-1474).

The methods for evaluating the presence of the
25 introduced DNA as well as its expression are readily available and well-known in the art. Such methods include, but are not limited to DNA (Southern) hybridization to detect the exogenous DNA, polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE) and Western blots
30 to detect DNA, RNA and protein.

Rabbits which possess sequences encoding human apolipoprotein (a) in its genomic DNA, including its germ line DNA, are mated with rabbits which possess human apolipoprotein B in its genomic DNA, including its germ line
35 DNA. In a specific embodiment, a transgenic rabbit that expresses apo-B can be mated with a transgenic rabbit that

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expresses physiological levels of apo(a), as described in WO 95/25793 and allowed U.S. Patent Application No. 08/704,582, now U.S. Patent No. 5,792,902, issued August 11, 1998, the specification of which is incorporated herein by reference in
5 its entirety. The resultant progeny are then screened to identify those rabbits which express apolipoprotein (a) and apolipoprotein B, such that lipoprotein (a) is produced.

As used herein, a "transgene" is a DNA sequence introduced into the germline of a non-human animal by way of
10 human intervention such as by way of the Examples described below.

Nucleic Acids

Transgenic animals according to the invention comprise nucleic acid sequences comprising genes encoding apo(a) and
15 apoB or variants thereof and are capable of expressing these genes to form a lipoprotein(a) complex. The nucleic acid may be of natural or artificial origin. It may be genomic DNA (gDNA), complementary DNA (cDNA), hybrid sequences or synthetic or semisynthetic sequences. It may be of human,
20 animal, plant, bacterial or viral origin and the like. It may be obtained by any technique known to persons skilled in the art, and especially by screening libraries, by chemical synthesis, or alternatively by mixed methods including chemical or enzymatic modification of sequences obtained by
25 screening libraries. It is preferably cDNA or gDNA.

The apo(a) gene, which is 1863 bp in length, has been cloned and sequenced (Sharpe et al., Nucleic Acids Res. (1984) 12(9):3917). In addition, a YAC clone containing the human apo(a) gene (clone 366H2 in the CEPH library) has
30 previously been described (Frazer, K. A., Narla, G., Zhang, J. L., & Rubin, E. M., Nat Gen (1995) 9, 424-431). To isolate the human apo(a) gene, the YAC can be separated from the yeast chromosomes by pulse field gel electrophoresis. The band containing the YAC is then excised from SeaPlaque
35 GTG agarose (FMC, Rockland, MD), treated with beta-agarase (NEB, Beverly, MA), and dialyzed against 0.1M NaCl, 10 mM

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Tris, 0.1 mM EDTA (Couto, L. B., Spangler, E. A., & Rubin, E. M., *Nuc Acid Res* (1989) 17(19), 8010).

A 90 kb P1 phagemid containing the human apoB gene has been previously described (Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) 91, 2130-2136). Alternatively, a DNA fragment containing the nucleic acid sequence encoding human apo(a) or apoB under the control of its own promoter can be obtained by screening human genomic or cDNA expression libraries. To do this, the library is screened by hybridization with a DNA probe which was complementary to human apolipoprotein(a) or apoB. The apo(a) or apoB DNA-containing clone(s) isolated in this screening are then analyzed by comparison with the published restriction maps for the AI/CII/AIV genomic complex (S.K. Karathanasis et al., *Proc. Natl. Acad. Sci. USA*, (1983) 80, 6147-6151; S.K. Karanthanasis, *Proc. Natl. Acad. Sci. USA*, (1985) 82, 6374-6378) or to their respective published sequences (Sharpe et al., *Nucleic Acids Res.* (1984) 12(9):3917; Frazer, K. A., Narla, G., Zhang, J. L., & Rubin, E. M., *Nat Gen.* (1995) 9, 424-431; and Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) 91, 2130-2136).

Regulatory Regions

Generally, the genes encoding apo(a) and apoB or variants thereof are linked to one or more regulatory regions. Selection of the appropriate regulatory region or regions is a routine matter that is within the level of ordinary skill in the art.

The regulatory regions may comprise a promoter region for functional transcription, as well as a region situated in 3' of the gene of interest, and which specifies a signal for termination of transcription and a polyadenylation site. All these elements constitute an expression cassette.

Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoter may be naturally

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responsible for the expression of the nucleic acid. It may also be from a heterologous source and may be promoter sequences of eucaryotic or viral genes. For example, it may be promoter sequences derived from the genome of the cell
5 which it is desired to infect. Likewise, it may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, HCMV and RSV genes and the like. In addition, the promoter may be modified by
10 addition of activating or regulatory sequences or sequences allowing a tissue-specific or predominant expression. Tissue specific promoters provide the means for modeling a variety of diseases. When the nucleic acid does not contain promoter sequences, one may be inserted.

15 Additional promoters useful for practice of this invention are the ubiquitous promoters HPRT vimentin, actin and tubulin the intermediate filament promoters desmin neurofilaments, keratin, and GFAP; the therapeutic gene promoters MDR, CFTR, and factor VIII promoters which are
20 preferentially activated in dividing cells; promoters which respond to a stimulus such as steroid hormone receptor and retinoic acid receptor promoters; tetracycline-regulated transcriptional modulators; cytomegalovirus immediate-early; retroviral LTR metallothionein; SV40, E1a and MLP promoters.
25 Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the contents of which are incorporated herein by reference.

In certain embodiments of the invention, the human
30 apo(a) and apoB genes or variants thereof are operably linked to a strong promoter, for example, a human cytomegalovirus (HCMV) promoter, so that high levels of transgene expression are achieved. A transgenic animal comprising such constructs would provide a model of human lipoprotein(a) expression and,
35 therefore, of atherosclerosis, stroke, ischemia, restenosis, cardiovascular disease, coronary artery disease, peripheral

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occlusive arterial disease, myocardial infarction, undesirable lipid profile, and thrombosis as well.

In another embodiment of the invention, the human apo(a) and apoB genes are operably linked to tissue specific promoter(s) in order to localize overexpression of the transgenes to particular cells. Preferred tissue specific promoters include vasculature and endothelial cells.

It is also possible to overexpress the human apo (a) and apo B genes operably linked to a sequence encoding a mutated ligand binding domain from a glucocorticoid or estrogen receptor fused to the genes. In this embodiment, expression of the genes is regulated by glucocorticoids or tamoxifen.

Vectors

As discussed above, a "vector" is any means for the transfer of a nucleic acid into a host cell. Preferred vectors are plasmids and viral vectors, such as, for example, retroviruses.

Viral vectors may be used to produce a transgenic animal according to the invention. Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors which are used within the scope of the present invention lack at least one region which is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), or be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed *in vitro* (on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents.

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Preferably, the replication defective virus retains the sequences of its genome which are necessary for encapsidating the viral particles.

The retroviruses are integrating viruses which infect
5 dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). The construction of recombinant retroviral vectors has been described: see, in particular, EP 453242, EP178220, Bernstein et al. Genet. Eng. (1985) 7:235; McCormick,
10 BioTechnology (1985) 3: 689 etc. In recombinant retroviral vectors, the gag, pol and env genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV
15 ("Moloney murine leukemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are disclosed in WO95/02697.

In general, in order to construct recombinant
20 retroviruses containing a nucleic acid sequence, a plasmid is constructed which contains the LTRs, the encapsidation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions which are deficient
25 in the plasmid. In general, the packaging cell lines are thus able to express the gag, pol and env genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (U.S. Patent No. 4,861,719); the PsiCRIP cell line (WO90/02806) and the GP+envAm-12 cell
30 line (WO89/07150). In addition, the recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences which may include a part of the gag gene (Bender et al., J. Virol. (1987) 61: 1639). Recombinant
35 retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

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Drug Screening Assays

Transgenic animals overexpressing the human apo(a) and apoB genes or variants thereof are useful in drug screening assays. These assays are useful for identifying compounds effective for the treatment of diseases, such as atherosclerosis, stroke, ischemia, restenosis, cardiovascular disease, coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, undesirable lipid profile, thrombosis and the like.

10 The present invention provides methods of determining the ability of a compound to modulate apo(a), apoB and lipoprotein(a) functions in a cell. Preferred methods comprise administering a compound to a transgenic non-human animal comprising the human apo(a) and apoB genes or variants thereof which are expressed and form a human lipoprotein(a) complex and comparing the potential therapeutic effects of the compound in the transgenic animals compared to that in control animals. Potential therapeutic compounds that prevent and/or treat the diseases or disorders that are associated with, induced and/or exacerbated by Lp(a) expression, for example, atherosclerosis, restenosis, stroke, ischemia, restenosis, cardiovascular disease, coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, undesirable lipid profile, and thrombosis and the like may be determined.

The present invention also relates to the use of the claimed transgenic rabbit for detecting the activity of therapeutic agents/compounds or therapeutic methods with a view to preventing and/or treating diseases linked to Lp(a) expression as well as to the methods for detecting novel compounds using this rabbit and to the compounds which are thus characterized. This transgenic rabbit animal model system is particularly advantageous for detecting specific therapeutic agents/compounds for treating and/or preventing diseases linked to Lp(a) expression and/or metabolism, in particular in the field of cardiovascular diseases such as atherosclerosis, cardiovascular disease, coronary artery

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disease, stroke, ischemia, peripheral occlusive arterial disease, restenosis, thrombosis, myocardial infarction, angina pectoris, sudden death, an undesirable lipid profile, cardiac decompensation or cerebrovascular diseases.

5 The invention also provides methods for identifying a compound effective for the treatment of diseases and disorders associated with, induced and/or exacerbated by Lp(a) expression, such as atherosclerosis, restenosis, stroke, ischemia, restenosis, cardiovascular disease,
10 coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, undesirable lipid profile, and thrombosis and the like. Such methods comprise administering a compound to a transgenic non-human animal comprising the human apo(a) and apoB genes or variants
15 thereof, wherein the transgenes are expressed and form a human lipoprotein(a) complex, and comparing the potential therapeutic effects of the compound in the transgenic animals compared to that in control transgenic animals.

 The present invention also provides a method for
20 identifying a compound that is able to modulate human Lp(a) assembly in a cell by administering a compound to a transgenic non-human animal comprising the human apo(a) and apoB genes or variants thereof, wherein the transgenes are expressed and form a human lipoprotein(a) complex, and
25 monitoring the human Lp(a) levels and/or biological activity within the treated transgenic animal as compared to that in control transgenic animals. In a preferred aspect of the invention, the cells are cells of an animal suffering from a disease or disorder associated with, induced and/or
30 exacerbated by Lp(a) expression and the method comprises administering to the cells of an animal a compound capable of modulating Lp(a) function to prevent and/or treat the disease/disorder. Such diseases include atherosclerosis, restenosis, stroke, ischemia, restenosis, cardiovascular
35 disease, coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, undesirable lipid profile, and thrombosis and the like.

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The drug screening assays of the invention may be performed on a large scale or small scale. Large scale screening involves visual observation of the vasculature or other tissue expressing the apo(a) and apoB transgenes, and
5 comparison to control animals. For example, if the human Lp(a) production within the transgenic animal results in atherosclerotic plaque formation in the blood vessels, the observer would evaluate a drug candidate based on its ability to decrease this formation relative to untreated animals.

10 Alternatively, the transgenic animals of the invention that express human Lp(a) or a variant thereof and also comprise a reporter gene operably linked to a promoter whose expression is altered by Lp(a) or a variant thereof may be prepared. For example, such a promoter operably linked to
15 the β -galactosidase gene (LacZ) would be uninduced or induced depending on the respective presence or absence of corrective treatment for Lp(a) expression.

Drug screening assays of the invention can also be performed on a small scale. Small scale assays involve
20 biopsy and either histological and/or immunohistochemical analyses. These characteristics can be evaluated histologically by a skilled practitioner. Immunohistochemical analysis can be performed using a marker for atherosclerosis, stroke, ischemia, restenosis, cardiovascular disease,
25 coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, undesirable lipid profile, thrombosis and the like.

Compounds capable of modulating Lp(a) function include those that are capable of inhibiting or down-regulating the
30 expression of either or both of the apo(a) and apoB genes, of inhibiting the formation of the Lp(a) complex or the biological activity of Lp(a) in a cell, or reducing the plasma level of lipoprotein(a). Such compounds include antisense nucleic acids, intracellular binding proteins,
35 including antibodies and any compound, natural or synthetic

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identified by using the transgenic animals and assays described herein.

Preparation and use of antisense polynucleotides, DNA encoding antisense RNA molecules and use of oligonucleotide
5 and genetic antisense are disclosed in WO 92/15680, the entire contents of which are incorporated herein by reference. Antisense nucleic acids for use according to the invention are preferably RNA capable of specifically hybridizing with all or part of the apo(a) and/or apoB genes,
10 or their corresponding messenger RNAs. The antisense sequence may be derived from DNA sequences whose expression in the cell produces RNA complementary to all or part of the apo(a) and/or apoB genes. These antisense sequences can be prepared by expression of all or part of a sequence encoding apo(a)
15 and/or apoB in the opposite orientation. Any length of the antisense sequence is suitable for practice of the invention so long as it is capable of down-regulating or blocking expression of the apo(a) and/or apoB genes. Preferably, the antisense sequence is at least 20 nucleotides in length.

20 In one aspect, the nucleic acid encodes antisense RNA molecules. In this embodiment, the nucleic acid is operably linked to suitable regulatory regions (discussed above) enabling expression of the nucleic acid sequence, and is introduced into a cell utilizing, preferably, recombinant
25 vector constructs, which will express the antisense nucleic acid once the vector is introduced into the cell. Examples of suitable vectors includes plasmids, adenoviruses, adeno-associated viruses (see WO 91/18088; WO 93/09239; U.S. Patent No. 4,797,368, U.S. Patent No. 5,139,941, EP 488 528),
30 retroviruses (see above), and herpes viruses. For delivery of a therapeutic gene, the vector is preferably an adenovirus.

Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the
35 invention to a variety of cell types.

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Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see WO94/26914). Those adenoviruses of animal origin which can be used within the scope of the present invention include adenoviruses of canine, bovine, murine, ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC VR-800), for example).

Preferably, the replication defective adenoviral vectors for use in the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (PvuII-BglII fragment) or 382 to 3446 (HinfII-Sau3A fragment). Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/28938), the E4 region (WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378, the contents of which are incorporated herein by reference. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted (see FR94 13355, the contents of which are incorporated herein by reference).

The replication defective recombinant adenoviruses can be prepared by any technique known to the person skilled in

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the art (Levrero et al., (1991) Gene 101, 195; EP 185 573; Graham, (1984) EMBO J. 3, 2917). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, inter alia, the DNA sequence of interest. The homologous recombination is effected following cotransfection of the said adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably (i) be transformable by the said elements, and (ii) contain the sequences which are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used are the human embryonic kidney cell line 293 (Graham et al., (1977) J. Gen. Virol. 36, 59) which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines which are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Another embodiment of the method of the invention to specifically inhibit Lp(a) activity comprises inhibiting Lp(a) function by expression of a nucleic acid sequence encoding an intracellular binding protein capable of selectively interacting with apo(a), apoB and/or the Lp(a) complex within a transfected cell. WO 94/29446 and WO 94/02610, the entire contents of which are incorporated herein by reference, disclose cellular transfection with genes encoding an intracellular binding protein. An intracellular binding protein includes any protein capable of selectively interacting, or binding, with apo(a), apoB and/or the Lp(a) complex, including human apo(a), apoB and/or Lp(a) in the cell in which it is expressed and of neutralizing the function of bound apo(a), apoB and/or Lp(a). Preferably, the intracellular binding protein is an antibody or a fragment of an antibody. Preferably, the intracellular binding protein is a single chain antibody. The antibody can be monoclonal

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or polyclonal.

WO 94/02610 discloses preparation of antibodies and identification of the nucleic acid encoding a particular antibody. Using the apo(a), apoB or Lp(a) complex proteins
5 or fragments thereof, a monoclonal antibody specific for apo(a), apoB or Lp(a) is prepared according to techniques known to those skilled in the art. A vector comprising the nucleic acid encoding an intracellular binding protein, or a portion thereof, and capable of expression in a host cell is
10 subsequently prepared for use in the method of this invention. Suitable vectors and methods of delivering nucleic acids encoding intracellular binding proteins to cells containing apo(a), apoB and/or Lp(a) include those discussed above for delivery of antisense nucleic acids.

15 In a preferred aspect of this embodiment, the nucleic acid sequence encoding an apo(a), apoB and/or Lp(a) intracellular binding protein additionally comprises a sequence encoding 1) a localization signal for targeting the intracellular binding protein to an appropriate cellular
20 location to permit interaction of the intracellular binding protein and its target molecule(s), and/or 2) a sequence enabling insertion of the intracellular binding protein in the plasma membrane. The localization signal or insertion sequence can be located anywhere on the intracellular binding
25 protein, so long as it does not interfere with the protein's ability to bind to apo(a), apoB and/or Lp(a). Examples of localization signals are disclosed in WO 94/02610.

Pharmaceutical Compositions

Compounds capable of modulating Lp(a) function include
30 antisense nucleic acids, intracellular binding proteins and compounds identified using the transgenic animals and assays described herein.

Antisense nucleic acid constructs that are capable of down-regulating or blocking expression of the apo(a) and apoB
35 transgenes may be delivered to affected cells. The nucleic

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acids, either in the form of a vector or naked DNA, may be combined with one or more pharmaceutically acceptable carriers for an injectable formulation. These may be in particular isotonic, sterile, saline solutions (monosodium or
5 disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, allow the constitution of injectable solutions.

10 The preferred sterile injectable preparations can be a solution or suspension in a nontoxic parenterally acceptable solvent or diluent. Examples of pharmaceutically acceptable carriers are saline, buffered saline, isotonic saline (e.g. monosodium or disodium phosphate, sodium, potassium, calcium
15 or magnesium chloride, or mixtures of such salts), Ringer's solution, dextrose, water, sterile water, glycerol, ethanol, and combinations thereof. 1,3-butanediol and sterile fixed oils are conveniently employed as solvents or suspending media. Any bland fixed oil can be employed including
20 synthetic mono- or diglycerides. Fatty acids such as oleic acid also find use in the preparation of injectables.

If administered as a virus the dose(s) may be adapted as a function of various parameters, and in particular as a function of the site of administration considered, the number
25 of injections, the gene to be expressed or alternatively the desired duration of treatment. In general, recombinant adenoviruses are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu, and preferably 10^6 to 10^{11} pfu. The term pfu (plaque forming unit) corresponds to the
30 infectivity of a virus solution, and is determined by infecting an appropriate cell culture and measuring, generally after 15 days, the number of plaques of infected cells. The technique for determining the pfu titre of a viral solution are well documented in the literature.

35 A nucleic acid, such as that encoding an antisense or intracellular binding protein, can also be administered as a

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naked DNA. Methods for formulating and administering naked DNA to mammalian muscle tissue are disclosed in US patents 5,580,859 and 5,589,466, the contents of which are incorporated herein by reference.

5

EXAMPLES

The present invention will be described in greater detail with the aid of the following examples which should be considered as illustrative and nonlimiting.

General Molecular Biology

- 10 In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A*
15 *Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization*
20 [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.),
25 *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

EXAMPLE 1 Human apo(a) and apo B DNA preparation and characterization

- The YAC clone containing the human apo(a) gene (clone
30 366H2 in the CEPH library) has previously been described (Frazer, K. A., Narla, G., Zhang, J. L., & Rubin, E. M., *Nat Gen* (1995) 9, 424-431; WO95/25793; allowed U.S. Patent Application No. 08/704,582). The YAC was separated from the yeast chromosomes by pulse field gel electrophoresis. The
35 band containing the YAC was excised from SeaPlaque GTG agarose (FMC, Rockland, MD), treated with beta-agarase (NEB, Beverly,

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MA), and then dialyzed against 0.1M NaCl, 10 mM Tris, 0.1 mM EDTA (Couto, L. B., Spangler, E. A., & Rubin, E. M., *Nuc Acid Res* (1989) 17(19), 8010). The 90 kb P1 phagemid containing the human apoB gene has been previously described (Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) 91, 2130-2136).

EXAMPLE 2 Production and screening of transgenic rabbits

The production of transgenic rabbits utilized the methods previously described by (Duverger, et al., *Arterioscler Thromb Vasc Biol* (1996) 16, 1424-1429). Briefly, New Zealand White adult female rabbits were superovulated, then mated on the same day that luteinizing hormone was injected. Embryos were collected 17 hours later. Embryo injection, transfer to pseudopregnant females and animal husbandry was as previously reported (Fan, et al., *Arterioscler Thromb Vasc Biol* (1995) 15, 1889-1899); Duverger, et al., *Arterioscler Thromb Vasc Biol* (1996) 16, 1424-1429). Approximately 2 pL of 1 ng/ml DNA solution containing either the human apo(a) YAC or the human apoB P1 phagemid was injected into the male pronuclei.

To create the apo(a) transgenic rabbits, 280 microinjected embryos were transferred into pseudopregnant females, resulting in 17 live-born rabbits. Rabbit DNA, extracted from ear biopsies, was screened for the presence of human apo(a) by PCR using methods and primers described previously (Frazer, et al., *Nat Gen* (1995) 9, 424-431). Two female rabbits were transgenic, and the animal with the highest apo(a) plasma level was used in subsequent studies. PCR screening was used to identify rabbits containing the transgenes and to confirm the complete integration of the apo(a) gene and its flanking regions (Figure 1). The apo(a) founder rabbits contained an intact copy of the entire YAC genomic clone, as determined by four different PCR reactions spanning the entire length of the clone. To create the apoB transgenics, 780 microinjected embryos were transferred into pseudopregnant females. Of the 20 live-born rabbits, two apoB transgenics were identified using ear biopsy DNA, PCR and human apoB specific primers from the promoter region of the gene: 5'-AGAAGGTTCCAGATGTCTATGAGG (SEQ ID No:1) and 5'-

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TCCAAGTATCTGTCTTCAAGAAACC (SEQ ID NO:2). The animal with the highest apoB plasma level was used in subsequent studies. The human apoB gene was detected by a single PCR reaction specific for human sequences in the promoter region. Offspring of the apo(a) and apoB founder animals were positive for the appropriate markers and demonstrated a Mendelian pattern of transmission of the two transgenes. Mating between the apo(a) and apoB transgenic founders produced rabbits of the predicted genotypes which are the subject of this invention.

10 EXAMPLE 3 Analysis of transgenic apo(a) and apo(b) tissue
distribution by RT-PCR

Total RNA was extracted from control and transgenic rabbit tissues using RNastat-60 (Tel-Test B, Friendswood, TX), subjected to DNase treatment, then reverse transcribed [5 µg RNA incubated with 10 ng apo(a)RT primer: 5' - GATGACCAAGCTTGGCAGGTTCTTCC - 3' (SEQ ID NO:3), or apoB M46 primer: 5' - CCACATTTTGAATCCAGGATGCAGTACTACT - 3' (SEQ ID NO:4) in a volume of 12 µl at 65°C for 10 minutes, then supplemented with Superscript II/buffer (GibcoBRL, Bethesda, MD) and incubated for 1 hour at 42°C]. The first strand cDNA was then treated with RNase, and extracted with phenol/chloroform. 5 µl of the RT product was subjected to PCR under standard conditions for Taq polymerase (Boehringer Mannheim, Indianapolis, IN) using 50 pmoles of each primer and thermocycling as follows: (94°C for 30 seconds, 62°C for 30 seconds, followed by 72°C for 90 seconds - 25 cycles). Apo(a) cDNA was detected using primers (a)Kr1F: 5' - ACCTGAGCAAAGCCATGTG - 3' (SEQ ID NO:5) and (a)Kr1R: 5' - AGTACTCCCACCTGACACCG - 3' (SEQ ID NO:6). ApoB cDNA was detected using human and rabbit specific PCR primers (M49/M50human or M49/M50rabbit, Hughes, S.D. et al., *Hum. Gene Ther.*, 7, 39-49 (1996)). PCR products were analyzed on 1.5% agarose gels. To demonstrate the absence of genomic DNA contamination, RNA preparations were directly subjected to the same PCR conditions, and no product was amplified.

To determine the distribution of apo(a) and apoB transgene expression, RNA preparations from several tissues

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(liver, testes, small intestine, kidney, brain, and lung) were subjected to RT-PCR assays as a qualitative determination of transgene expression (Figure 2). Apo(a) RNA was detected in the liver and the testes, while under more sensitive

5 conditions (increased number of PCR cycles from 25 to 30), it was also observed in the brain. The liver and testes were also the major sites of expression for the human apoB transgene. A relatively weak signal (< 5% of signal in liver) was detected in the small intestine. As a control for the

10 species specificity of the apoB mRNA assay, as well as for RNA quality, the RT-PCR assay was used to detect specifically rabbit apoB. As expected, rabbit apoB mRNA was detected in all tissues examined. Consistent with studies in transgenic mice, both human apo(a) and apoB transgenes were expressed at

15 significant levels and in the appropriate tissues in all founder rabbits identified, demonstrating the high efficiency and tissue specific expression of these genomic transgenes as compared to cDNA transgenes (Chiesa, et al., *J Biol Chem* (1992) **267**, 24369-24374; Chiesa, et al., *J Bio Chem* (1993) **268**, 23747-23750).

20

The tissue distribution of human apoB expression was somewhat different to that observed in transgenic mice. The low level human apoB intestinal expression in the transgenic rabbits indicates some basal level of transcription from the

25 human apoB promoter occurs in rabbit intestine, but the expression is far below that of endogenous apoB in the intestine, suggesting that control elements required for expression in this tissue were not present in the apoB genomic construct. This later point is consistent with the

30 conclusions of two independent reports of mice containing an identical apoB transgene (Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) **91**, 2130-2136; Purcell-Huynh, et al., *J Clin Invest* (1995) **95**, 2246-2257). In addition, we did not detect human apoB-48 in the plasma of transgenic rabbits. This

35 result, consistent with findings of (Fan, et al., *Arterioscler Thromb Vasc Biol* (1995) **15**, 1889-1899) suggests that little or no human apoB protein production occurs in the intestine of the transgenic rabbits.

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Because of the profound decrease in apo(a) plasma levels associated with sexual maturity previously noted in mice containing the apo(a) genomic transgene, we examined the effect of sexual maturity on apo(a) plasma levels in the transgenic rabbits. Plasma samples collected from rabbits at 3, 6, and 9 months of age (rabbits are sexually mature at 6 months of age) were evaluated for changes in apo(a) levels by immunoblotting (Figure 3). This analysis revealed no significant change in apo(a) levels over the course of sexual maturation in the transgenic rabbits.

While the tissue specificity of apo(a) genomic transgene expression was similar in rabbits and mice, developmental regulation of expression differed significantly between these two organisms. Unlike the striking effects of sexual maturity on apo(a) plasma levels in transgenic mice, apo(a) levels in rabbits were not altered by sexual maturation. Since plasma levels of apo(a) are primarily determined by synthetic rate, lack of a decline in apo(a) plasma levels accompanying sexual maturity in rabbits containing the apo(a) transgene suggests that sex hormones do not have a potent effect on transgene expression in these animals. Effects of these hormones on apo(a) transcription in rabbits may be more subtle, as has been noted in humans.

EXAMPLE 4 Apolipoprotein and Lipoprotein Analysis

The total lipoprotein fraction ($d < 1.21$ g/ml) was prepared from rabbit plasma samples by a density gradient ultracentrifugation method described previously (Callow, et al., *Proc Nat'l Acad Sci, USA* (1994) 91, 2130-2136). Lp(a) and human apoB concentrations were measured in rabbit plasma and lipoprotein fractions by a sandwich ELISA assay. For Lp(a), purified IgG prepared from goat polyclonal antiserum to human apolipoprotein (a) (International Immunology Corp., Murrieta, CA) was bound to the microtiter plate well as a capture antibody. A horseradish peroxidase conjugate of the same antibody was used for detection of apo(a) after addition of the chromogenic substrate o-Phenylene diamine. Standardization of the method was based on in-house human

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lipoprotein calibrators independently measured using a commercially available standardized Lp(a) ELISA kit (Strategic Diagnostics, Newark, DE). The assay for human apoB (B48 + B100) employed a mouse anti-human apoB antibody (Genzyme, Cambridge, MA). Standard curves were calculated with appropriate blanks and controls using Logit-Log data transformation. All reported concentrations were calculated from triplicate analysis.

To assess apo(a) and apoB interactions, plasma isolated from the transgenic rabbits was separated by SDS-PAGE on 4% polyacrylamide gels according to (Laemmli, E. K., *Nature* (1970) **227**, 680-685) and proteins were transferred to a Millipore nitrocellulose membrane by immunoblotting according to (Towbin, et al., J., *Proc Nat Acad Sci, USA* (1979) **6**, 4350-4354). Where indicated, samples were reduced by the addition of denaturing sample buffer containing 2.5% 2-mercaptoethanol and incubated at 100°C for 5 minutes. Apo(a) was detected with goat anti-apo(a) (Valbiotech, Paris) followed by HRP-conjugated rabbit anti-goat (BioRad, Hercules, CA). Human apoB was detected using HRP-conjugated polyclonal rabbit anti-human apoB (kindly provided by C. Fievet). Protein bands were revealed by an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and scanned using a Hoefer GS transmittance scanning densitometer. The area of the main peaks were used to evaluate the relative proportion of free and apoB-bound apo(a) (Gabel, et al., *Arterio. Thromb. Vasc. Biol.* (1996) **16**, 1559-1567). The apparent molecular weights were determined by the use of prestained standards (BioRad). Control human serum was used as positive control for expression of the human proteins.

Under non-reducing conditions, plasma samples from both the apo(a) transgenic rabbits and the combined human apoB/apo(a) transgenic rabbits demonstrated two bands (Figure 4A); the higher molecular weight band corresponding to the apoB/apo(a) complex, and the lower molecular weight band corresponding to free apo(a). The presence of a high molecular weight apo(a) band indicated assembly of Lp(a) in

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the combined transgenic rabbits, as well as some degree of covalent interaction between apo(a) and rabbit apoB in rabbits transgenic for apo(a) only. This result was confirmed by immunoblotting reduced plasma samples (Figure 4B), where only
5 a single band representing apo(a) was detected in both types of transgenic rabbits using the same human apo(a) antiserum. The apparent molecular weight of the apo(a) in the transgenic rabbits was approximately 200 kD. This is similar to the apo(a) size observed in the transgenic mice containing this
10 same apo(a) genomic construct, corresponding to an apo(a) protein containing approximately nine kringle 4 - like repeats. Samples blotted with human specific apoB antiserum (Figure 4C) gave a single band of 550 kD, representing human apoB-100, exclusively in the plasma of rabbits which tested
15 positive by PCR for human apoB. Concentrations of apo(a) and apoB in the transgenic rabbits were evaluated by ELISA. The average concentration of human apoB in the transgenic rabbits was 17.6 mg/dl. The average total apo(a) concentration in plasma was 2.5 mg/dl, with no significant difference between
20 the apo(a) only and human apoB/apo(a) transgenics.

Ultracentrifugation was used to examine the association of apo(a) with the lipoprotein fraction. The disulfide linkage between apo(a) and human apoB has been shown to withstand the conditions of ultracentrifugation used to
25 isolate lipoproteins, while non-covalently associated apo(a) fractionates with the more dense plasma proteins (Utermann, G., & Weber, W., *FEBS Let* (1983) 154, 357-361). Lipoprotein fractions isolated from apo(a) transgenics and apoB/apo(a) transgenic rabbits were tested for the presence of apo(a) by
30 ELISA. Apo(a) was detected in the lipoprotein fractions of both types of transgenic rabbits, indicating apo(a) formed a disulfide bond between both human and rabbit apoB. The relative proportions of Lp(a) and apo(a) in the plasma, however, suggested that the rabbit apoB-apo(a) complex was
35 formed *in vivo* at a relatively lower efficiency than that observed with human apoB. Comparing the patterns of apo(a) immunoreactivity between apo(a) transgenic and apoB/apo(a) transgenic rabbits (Figure 4A) revealed a greater proportion

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of non-covalently bound apo(a) when only rabbit apoB was present. The relative ratios of the different molecular forms of apo(a) in the transgenic rabbits were estimated from immunoblots by densitometric scanning of chemiluminescent
5 films. In apoB/apo(a) transgenics, approximately 20% of the apo(a) consistently migrated as free apo(a) (n=3), with the remaining 80% covalently bound to either human or rabbit apoB. In apo(a) transgenic rabbits, the reverse distribution was observed (80% free protein and 20% bound to rabbit apoB; n=3).
10 Although these ratios are not rigorously quantitative, the consistent differences between the two groups of transgenic rabbits containing the same apo(a) transgene and similar total apo(a) levels suggest that apo(a) forms a disulfide linkage more efficiently with human apoB than rabbit apoB.

15 The ability of rabbit apoB to form a covalent linkage with apo(a) was first suggested by *in vitro* studies examining human apo(a) interactions with apoB from a number of mammals which demonstrated that rabbit apoB may form a covalent interaction with human apo(a) under certain conditions (Trieu,
20 V., & McConathy, W. J., *Biochem. J.* (1995) 309, 899-904). The demonstration of Lp(a) assembly in the rabbits expressing only the apo(a) transgene substantiates this interaction under *in vivo* conditions. In comparison, rabbits containing both human apoB and apo(a) transgenes contained a much higher
25 proportion of apo(a) in the Lp(a) form. However, despite adequate levels of human apoB in the combined apoB/apo(a) transgenic rabbits, approximately 20% of the apo(a) persisted in a non-covalently bound state. This is in contrast to the condition in humans where all the apo(a) is covalently bound
30 to apoB, and the presence of free apo(a) has been observed only in rare cases of abetalipoproteinemia (Menzel, et al., *J Bio Chem* (1990) 265, 981-986).

EXAMPLE 5 Fractionation of plasma lipoproteins and cholesterol measurement

35 Freshly isolated plasma was fractionated by gel permeation chromatography using a Superose 6 column (Pharmacia). 100 μ l of plasma was injected onto the column and

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run under isocratic conditions with a flow rate of 40 μ l/min in GF2 buffer (20 mM Tris pH 8.0, 0.27 mM EDTA, 150 mM NaCl). The effluent was monitored for absorbance at 280 nm, and 50 μ l fractions were collected during the run and subsequently
5 assayed for cholesterol using a commercial cholesterol assay kit (Boehringer-Mannheim). A 20 μ l aliquot of cholesterol-containing fractions was used to detect the presence of apo(a) and Lp(a) using electrophoresis and immunoblotting as described above.

10 To further characterize the association of apo(a) with lipoproteins in the transgenic rabbits, lipoproteins were size-fractionated by gel filtration chromatography. In contrast to density gradient centrifugation methods, chromatographic methods can isolate lipoprotein complexes
15 containing proteins associated by weak, non-covalent interactions. Immuno-blotting of plasma fractionated in this manner showed that apo(a) was present primarily in the LDL fraction in both human apoB/apo(a) (Figure 5A) and apo(a) transgenic rabbits (Figure 5B). LDL fractions of combined
20 transgenic rabbits contained two distinct high molecular weight bands corresponding to the human apoB/apo(a) complex and rabbit apoB/apo(a) complex, distinguished on the basis of the lower molecular weight of rabbit apoB-100 (320 kD). Non-covalently associated apo(a), although present in the
25 immunoblot of total plasma, was not detected in the LDL fractions of human apoB/apo(a) transgenic rabbits. LDL fractions of apo(a) transgenic rabbits contained a broad band representing rabbit apoB/apo(a) complex accompanied by a relatively larger amount of apo(a) dissociated from LDL under
30 electrophoresis conditions, designated as free apo(a) (Figure 5B). This analysis confirmed the association of apo(a) with lipoproteins containing both human and rabbit apoB, although in the case of rabbit apoB, apo(a) was linked through a disulfide bond in a much lower proportion of particles.

35 In the present invention, apo(a) exhibited specific non-covalent interactions with apoB-containing lipoproteins, suggesting a highly conserved affinity between apo(a) and

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apoB, similar to results of previous studies of apo(a) interactions with apoB of several other species (Chiesa, et al., *J Bio Chem* (1993) **268**, 23747-23750; Trieu, V., & McConathy, W. J., *Biochem. J.* (1995) **309**, 899-904). This is supported by the observation of non-covalently associated apo(a) in the LDL fraction of apo(a) transgenic rabbits. Such non-covalent interactions between apo(a) and apoB have been proposed to facilitate Lp(a) assembly by bringing cysteine residues in position for disulfide bond formation (Trieu, V., & McConathy, W. J., *J. Biol. Chem.* (1995) **270**, 15471-15474). In the plasma of the apoB/apo(a) transgenic rabbits, apo(a) may persist in this non-covalently bound form due to the capacity of rabbit apoB to effectively trap some apo(a) in the initial binding step, while disulfide bond formation from this intermediate proceeds with much lower efficiency since covalent linkage must occur between cysteines which are not optimally positioned through non-covalent interactions.

EXAMPLE 6 Sequence comparison of putative apo(a) binding site in human and rabbit apoB

Due to the surprising ability of rabbit apoB, unlike murine apoB, to form a covalent linkage with human apo(a), we made a comparison of its amino acid sequence within a region homologous to the site of apo(a) attachment in human apoB. Since the relevant portion of rabbit apoB has not been reported, the carboxy-terminal region of a rabbit apoB cDNA clone was sequenced. The derived protein sequence for rabbit apoB is shown in Figure 6 aligned within a region of significant homology to four other reported apoB sequences, including human (SEQ ID NOS:7-11). As in rodents and pigs, two mammals in which apoB has been demonstrated unable to form a covalent linkage with human apo(a) *in vitro*, rabbit apoB also lacks a cysteine at a position homologous to the site of apo(a) attachment in human apoB (cys4326). These results, in contrast to two previous reports suggesting that a cysteine residue at this position is required for covalent interaction with apo(a) (Callow, M. J., & Rubin, E. M., *J Biol Chem* (1995) **270**, 23914-23917; McCormick, S. P. A., Ng, J. K.,

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Taylor, S., Flynn, L. M., Hammer, R. E., & Young, S. G., (1995) 92, 10147-10151), indicate that cysteines elsewhere in the rabbit apoB molecule are capable of covalent interaction with apo(a).

- 5 All the references discussed herein are incorporated by reference.

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as
10 those inherent therein. The transgenic animals, methods, procedures and techniques described herein are presented as representative of the preferred embodiments, and intended to be exemplary and not intended as limitations on the scope of the present invention. Changes therein and other uses will
15 occur to those of skill in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

CLAIMS

We claim:

1. A transgenic rabbit which has in its genomic DNA sequences that encode apolipoprotein (a) and apolipoprotein B polypeptides which are capable of combining to produce lipoprotein (a).
2. The rabbit of Claim 1 wherein said rabbit develops human-like atherosclerotic lesions when fed a cholesterol rich diet.
3. The rabbit of Claim 1 having exogenous human DNA sequences.
4. The rabbit of Claim 2 having exogenous human DNA sequences.
5. The rabbit of Claim 1 having exogenous DNA sequences selected from the group consisting of genomic DNA and cDNA.
6. The rabbit of Claim 2 having exogenous DNA sequences selected from the group consisting of genomic DNA and cDNA.
7. The rabbit of Claim 1 having liver cells which express said sequences.
8. The rabbit of Claim 2 having liver cells which express said sequences.
9. The rabbit of Claim 1 having testes cells which express said sequences.
10. The rabbit of Claim 2 having testes cells which express said sequences.

11. The rabbit of Claim 1 having a stable plasma level of apolipoprotein (a) polypeptide throughout its sexual maturity.

12. A process for creating a transgenic rabbit which is capable of expressing apolipoprotein (a) and apolipoprotein B polypeptides and which comprises combining the germ line cells of a first rabbit which is capable of expressing apolipoprotein (a) with the germ line cells of a second rabbit which is capable of expressing apolipoprotein B.

13. A process for creating a transgenic rabbit which is capable of expressing apolipoprotein (a) and apolipoprotein B polypeptides and which comprises mating a first rabbit which is capable of expressing apolipoprotein (a) with another rabbit which is capable of expressing apolipoprotein B.

14. The process of Claim 13 wherein said rabbit capable of expressing apolipoprotein B protein is provided by injecting a rabbit embryo with a phagemid which contains the human apolipoprotein B gene.

15. The method of Claim 13 wherein said rabbit capable of expressing apolipoprotein (a) is provided by injecting a rabbit embryo with a yeast artificial chromosome which contains a human apolipoprotein (a) gene.

16. The method of Claim 13 wherein said rabbit capable of expressing apolipoprotein (a) is provided by introducing into a rabbit embryonal stem cell at least one human DNA fragment encoding apolipoprotein (a) protein, combining said stem cell with a rabbit blastocyst and transferring the embryo to a recipient female rabbit.

17. The method of Claim 13 wherein said rabbit capable of expressing apolipoprotein B is provided by introducing into a rabbit embryonal stem cell at least one human DNA fragment encoding apolipoprotein B protein, combining said

stem cell with a rabbit blastocyst and transferring the embryo to a recipient female rabbit.

18. The method of Claim 13 wherein said rabbit expressing apolipoprotein (a) is provided by infecting a rabbit blastomere with a retrovirus comprising at least one human DNA fragment encoding apolipoprotein (a) protein and transferring the blastomere to a recipient female rabbit.

19. The method of Claim 13 wherein said rabbit expressing apolipoprotein B is provided by infecting a rabbit blastomere with a retrovirus comprising at least one human DNA fragment encoding apolipoprotein B protein, and transferring the blastomere to a recipient female rabbit.

20. A method for determining whether a compound can inhibit a disease or disorder linked to lipoprotein (a) expression or metabolism comprising comparing a first transgenic rabbit which is capable of producing human lipoprotein (a) and manifests a disease or disorder linked to human lipoprotein (a) and which has been treated with said compound, with a second transgenic rabbit which is capable of producing human lipoprotein (a) and manifests a disease or disorder linked to lipoprotein (a) and which has not been treated with said compound.

21. The method according to Claim 20, wherein the disease or disorder is selected from the group consisting of atherosclerosis, cardiovascular disease, ischemia, stroke, restenosis, coronary artery disease, peripheral occlusive arterial disease, myocardial infarction, thrombosis, and an undesirable lipid profile.

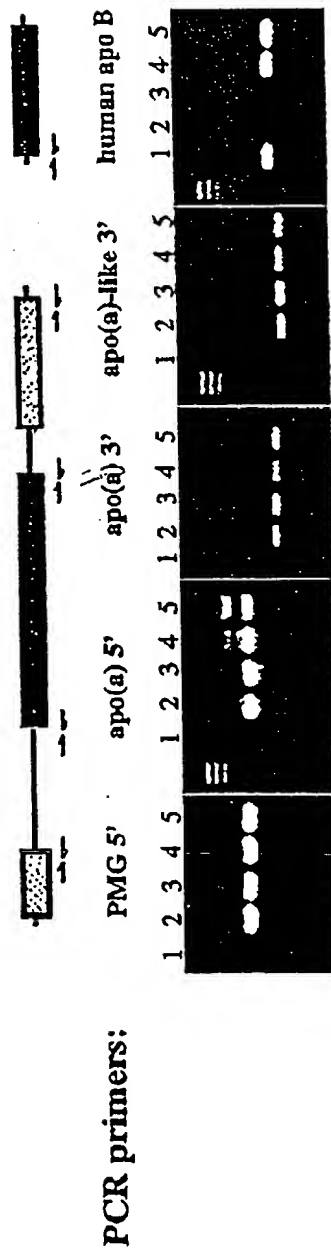
22. A method for determining whether a compound can inhibit assembly of a lipoprotein (a) particle comprising comparing the level of lipoprotein (a) production in a first transgenic rabbit which has been treated with said compound and which is capable of producing human lipoprotein (a) to the level of production of lipoprotein (a) in a second

transgenic rabbit that has not been treated with said compound and which is capable of producing human lipoprotein (a).

23. The method of Claim 22 wherein said compound is selected from the group consisting of antisense nucleic acids, and intracellular binding proteins.

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Figure 1



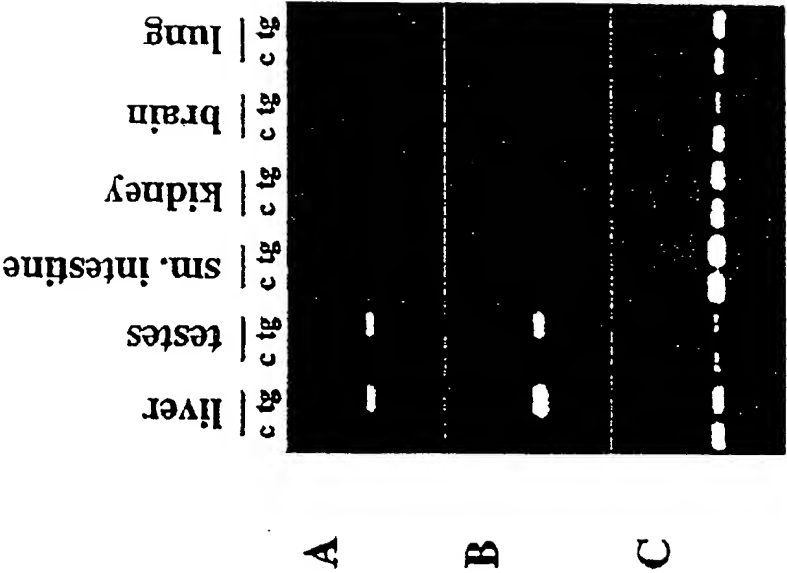
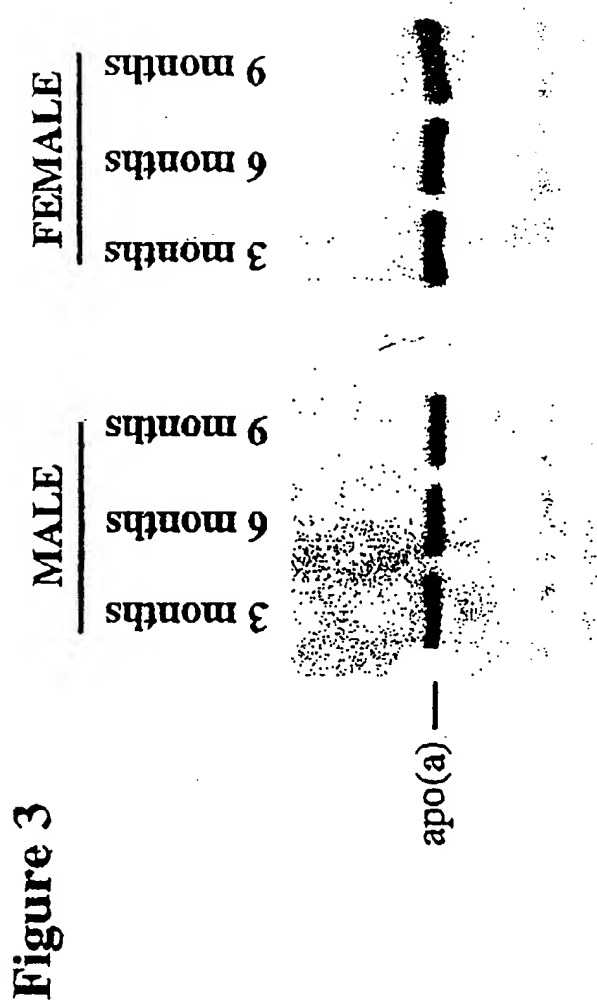


Figure 2

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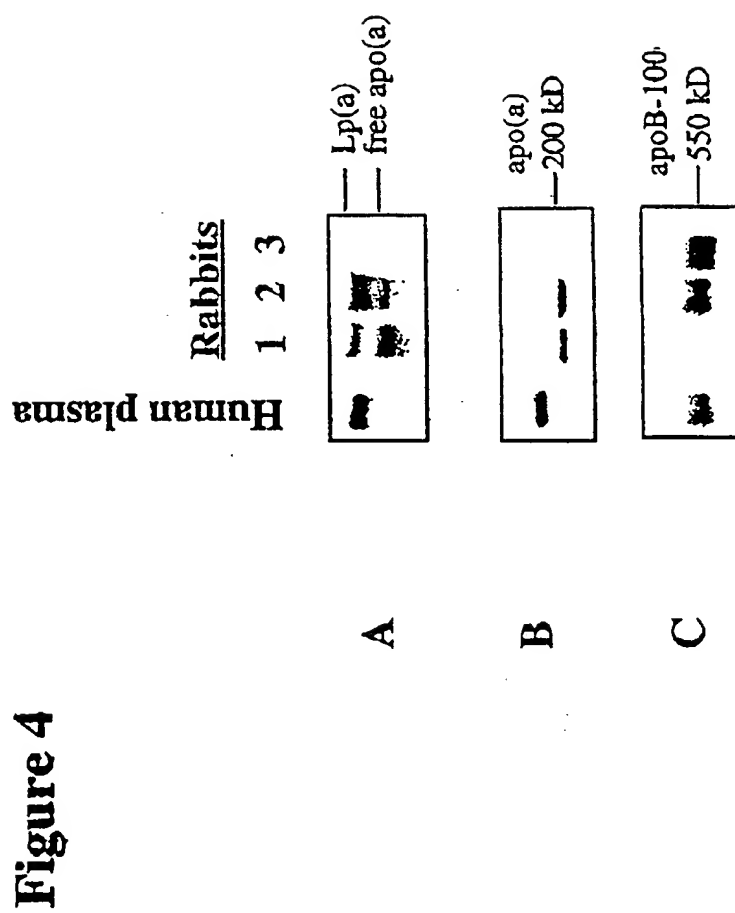
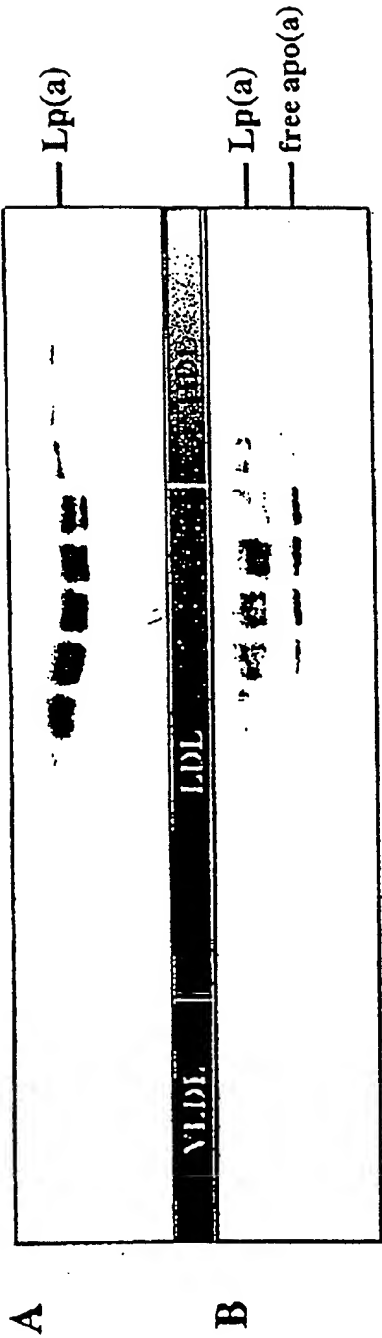


Figure 5



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Figure 6

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		*	
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rat	pfaFksLrEN	iysvfsefND	FvQsiliQEgS yKLQQvHQYn KAfREEYFDP
pig	PlgFRllKEN	LdspfgmlNE	FiQNTLWEAS QELQQiHQYI KALRkEYFDP
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SEQUENCE LISTING

<110> Rouy, Didier
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Emmanuel, Florence
Denefle, Patrice
Houdebine, Louis-Marie
Viglietta, Celine
Rubin, Edward M.
Hughes, Steven D.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00401

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 9/00, 11/10, 15/00; A61K 49/00; G01N 31/00

US CL : 800/9, 13, 21, 22, 25; 435/178, 183, 320.1; 424/9.1, 9.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/9, 13, 21, 22, 25; 435/178, 183, 320.1; 424/9.1, 9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P --- Y,P	US 5,792,902 A (BENOIT et al) 11 August 1998, claims 11-21.	12, 20-22 ----- 1-8, 11, 13-19, 23
Y	US 5,434,058 A (DAVIDSON) 18 July 1995, claims 1-9.	1-8, 11-23
Y	HEIDENREICH et al. Application of antisense technology to therapeutics. Molecular Medicine Today. June 1995, Vol. 1, No. 3, pages 128-133, especially abstract.	22, 23

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 23 MARCH 1999	Date of mailing of the international search report 15 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CATHERINE STROUP Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00401

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROUY et al. Apolipoprotein (a) Yeast Artificial Chromosome Transgenic Rabbits. J. Biological Chemistry. 09 January 1998, Vol. 273, No. 2, pages 1247-1251, especially abstract.	1-8, 11-19
X	DUVERGER et al. Inhibition of Atherosclerosis Development in Cholesterol-Fed Human Apolipoprotein A-I-Transgenic Rabbits. Circulation. 15 August 1996, Vol. 94, No. 4, pages 713-717, especially abstract and last paragraph.	1-8, 11, 12, 20-22
X	LEBUFFE et al. Endothelial Derived Vasorelaxation Is Impaired in Human APO A-I Transgenic Rabbits. Biochem. Biophys. Res. Comm. December 1997, Vol. 241, pages 205-211, especially abstract.	1-8, 11, 12
T,P	BROUSSEAU et al. Transgenic rabbits as models for atherosclerosis research. J. Lipids Res. March 1999, Vol. 40, pages 365-375, especially abstract.	1-23